GUINEA PIGS SUBLETHALLY INFECTED WITH AEROSOLIZED *LEGIONELLA PNEUMOPHILA* DEVELOP HUMORAL AND CELL-MEDIATED IMMUNE RESPONSES AND ARE PROTECTED AGAINST LETHAL AEROSOL CHALLENGE

A Model for Studying Host Defense against Lung Infections Caused by Intracellular Pathogens

By ROBERT F. BREIMAN AND MARCUS A. HORWITZ

From the Division of Infectious Diseases, Department of Medicine, University of California, Los Angeles School of Medicine, Los Angeles, California 90024

Legionella pneumophila is a facultative intracellular bacterial pathogen (1) and the agent of Legionnaires' disease, a serious and often fatal form of pneumonia. The organism is spread to humans from contaminated sources by the airborne route. Patients with Legionnaires' disease develop both humoral and cell-mediated immune responses (2–4). Humoral immune responses appear to play a secondary role in host defense because antibody does not promote killing of *L. pneumophila* by complement, promotes only modest killing of *L. pneumophila* by phagocytes (polymorphonuclear leukocytes, monocytes, or alveolar macrophages), and does not inhibit intracellular multiplication in monocytes. Cell-mediated immune responses appear to play a primary role in host defense because activated monocytes and alveolar macrophages inhibit *L. pneumophila* intracellular multiplication (4–6).

The guinea pig, which shares with humans a susceptibility to lung infection with *L. pneumophila*, is an excellent animal model for the study of Legionnaires' disease (7–11). When guinea pigs are exposed to aerosols containing *L. pneumophila*, they develop, after an incubation period of a few days, a pneumonic illness characterized by fever, weight loss, and labored respirations that sometimes culminates in death. This syndrome strongly resembles Legionnaires' disease in humans both clinically and pathologically (7–9).

Sublethal exposure of guinea pigs to *L. pneumophila* has not been demonstrated to result in protective immunity to lethal challenge with this organism in previous studies. Two studies reported no protection against lethal challenge after sublethal exposure (12, 13). This seemed surprising in view of what was known about host defense against this organism.

In this study, we have evaluated the immune responses of the guinea pig to

This work was supported by grant AI-22421 from the National Institutes of Health. Dr. Horwitz is a Gordon MacDonald Scholar at University of California, Los Angeles and a recipient of a faculty research award from the American Cancer.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/87/03/0799/13 $1.00
Volume 164 March 1987 799–811

sublethal aerosol exposure with *L. pneumophila* and examined the protective effects of such exposure. We shall demonstrate that guinea pigs sublethally infected with *L. pneumophila* by aerosol (*a*) develop humoral immune responses, (*b*) develop cell-mediated immune responses and cutaneous delayed-type hypersensitivity, (*c*) are protected against subsequent lethal aerosol challenge with *L. pneumophila*, and (*d*) are able to limit *L. pneumophila* multiplication when challenged and to clear the bacteria from their lungs.

Materials and Methods

Media. RPMI 1640 with L-glutamine (Gibco Laboratories, Grand Island, NY), egg yolk buffer (EYB)[1] with (EYB-BSA) or without 1% BSA, and Dulbecco's PBS with Ca^{2+} and Mg^{2+} ions were obtained or prepared as described previously (1, 14).

Agar. Modified charcoal yeast extract (CYE) agar was prepared as described (1).

Bacteria. *L. pneumophila,* Philadelphia 1 strain, was grown in embryonated hens' eggs, harvested, tested for viability and the presence of contaminating bacteria, as described (1), passed one time only on CYE agar, washed with EYB, flash frozen in aliquots of 10^{11} CFU/ml, and stored at −70°C. Before use in the aerosol inoculation system, each stock preparation was diluted in EYB to the desired concentration. Formalin-killed *L. pneumophila* was prepared as previously described (4).

Animals. Male Hartley strain guinea pigs weighing 250–300 g, purchased from Charles River Breeding Laboratories (North Wilmington, MA), were housed three to a stainless steel cage and allowed free access to ascorbic acid–fortified chow and water before the experiment. They were observed in the vivarium for signs of illness for ~1 wk before each experiment to ensure that they were healthy at the start of the experiment. After they were exposed to aerosols, the guinea pigs were kept in filter top cages for 1 wk. They were observed for signs of illness, their rectal temperatures were taken with a Telethermometer with rectal probe (Yellow Springs Instrument Co., Yellow Springs, OH), and their weights were recorded daily. They were allowed free access to chow and water throughout the experiment.

Aerosol Inoculation System. This system was constructed with the guidance and generous assistance of Paul Edelstein (Wadsworth Veterans Administration Medical Center, Los Angeles, CA). The aerosol chamber was constructed of lucite and measured 13 × 24 × 18 in. On opposite sides it had two 6-in-diam portals for introducing or removing guinea pigs; these sealed tightly during aerosolization. The aerosol inlet was located at the center of the chamber ceiling; it was connected to a Dart aerosol nebulizer system (Dart Industries, Ocala, FL). A vacuum pump (Gast Mfg. Co., Benton Harbor, MI) delivered positive pressure to the nebulizer and applied negative pressure to the chamber via a small outlet valve. Although the chamber was self-contained, it was placed within a laminar flow hood during aerosolization as an extra measure of safety. Guinea pigs were placed inside of the chamber and 10 ml of a suspension of *L. pneumophila* or control buffer was placed in the nebulizer reservoir. The vacuum pump was then turned on, generating an aerosol into the chamber. Aerosolization was allowed to continue for 30 min or until the nebulizer was empty (whichever came first). The pump was then turned off and the guinea pigs were held within the device for 10 min. The animals were then removed and placed in filter top cages, one to three to a cage. When shared, cages contained only guinea pigs that had been exposed to the same concentration of *L. pneumophila*.

The number of CFU of *L. pneumophila* in the lungs of guinea pigs after exposure to aerosols was only a small fraction of the number aerosolized. In two experiments, four guinea pigs exposed to an aerosol of 10^8 CFU/ml had an average of 2.0×10^4 CFU in their lungs 1 h later.

Serology. Antibody against *L. pneumophila* in the serum of selected guinea pigs was determined by the indirect fluorescent antibody (IFAb) technique as described for human

[1] *Abbreviations used in this paper:* CYE, modified charcoal yeast extract; EYB, egg yolk buffer; IFAb, indirect fluorescent antibody.

serum by Wilkinson et al. (15). Antigen-bearing slide wells were prepared by covering each well of a 12-well microscope slide (Cel-Line Associates, Minotola, NJ) with 30 μl of a 2×10^9 cells/ml preparation of formalin-killed *L. pneumophila*, allowing the slides to air dry, fixing the slides in acetone for 15 min, and again allowing the slides to air dry. Serum was obtained from the animals by cardiac puncture just before sacrifice, serially diluted in an equal volume of PBS up to a concentration of 1:1,024, and placed on an antigen-bearing slide well. The slides were incubated at 37°C in 100% humidity for 30 min, rinsed in PBS, and soaked in a PBS bath for 10 min. Then, 20 μl of fluorescein-conjugated IgG fraction goat anti–guinea pig IgG (Fc fragment) (Cappel Laboratories, Cochranville, PA) was placed on each well, and the slides were incubated at 37°C in 100% humidity for 30 min and rinsed. The slides were viewed at × 1,000 with a fluorescence microscope (Nikon Inc., Garden City, NY). The titer of each animal's serum was expressed as the reciprocal of the dilution which produced 2+ fluorescence staining of at least 50% of the *L. pneumophila* bacteria in a microscopic field.

Cutaneous Delayed-type Hypersensitivity. Guinea pigs were shaved over the back and flank and given intradermal injections at different sites in this area of various concentrations of formalin-killed *L. pneumophila* diluted in 0.1 ml of PBS. The diameter of areas of erythema and induration at the site of the injection was recorded at 24 and 48 h.

Lymphocyte Proliferation Assay. Guinea pigs were killed by hypercarbia. The bodies were soaked in Linbro 7X cleaning solution (Flow Laboratories, Inc., McClean, VA) and the spleens were removed using sterile technique. Spleens were placed in RPMI 1640 with penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco) and ground to a pulp. The pulp was strained through a thin layer of nylon mesh to remove fibrous material, and the spleen cells in the liquid passing through the mesh were collected by centrifugation at 200 *g* for 10 min at 4°C. Residual erythrocytes were hypotonically lysed and isotonicity was restored as described (16). The remaining splenic lymphocytes were washed by centrifugation and counted in a hemocytometer. For the lymphocyte proliferation assay, the lymphocytes were adjusted to a final concentration of 10^7/ml in RPMI 1640 containing penicillin (100 U/ml), streptomycin, (100 μg/ml), and 10% FCS and incubated with various concentrations of formalin-killed *L. pneumophila* as antigen in a total volume of 100 μl in microtest wells (96-well flat-bottom tissue culture plate, Falcon Labware, Oxnard, CA). As controls, some lymphocytes were incubated without antigen, and others were incubated with Con A. The lymphocytes were incubated at 37°C in 5% CO_2/95% air for 1–5 d. At the end of this incubation period, 0.25 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA) was added to each well and the cells were incubated at 37°C in 5% CO_2/95% air for 2 h. Using a multisample automated cell harvester (Skatron, Inc., Sterling, VA), each well was washed with double-distilled water and the effluent was passed through a filtermat. Filtermat sections representing separate microtest wells were placed in scintillation vials and 2 ml of biofluor liquid scintillation cocktail (New England Nuclear) was added. After at least 24 h, the beta particle emission was measured in a beta scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Determination of Lowest Lethal Dose (LD). *L. pneumophila*, prepared as described above, was diluted in EYB to concentrations of 10^6, 10^7, 5×10^7, 7.5×10^7, 10^8, 2.5×10^8, 5×10^8, and 10^9 CFU/ml. Groups of animals were exposed in the aerosol delivery system to aerosolized 10-ml aliquots of each of these concentrations of *L. pneumophila*. Over the subsequent week, the rectal temperature, weight, and any physical signs of illness such as lethargy, ruffled fur, labored respiration, grunting and death were recorded daily on each animal.

Quantitation of L. pneumophila *in Pulmonary Tissue.* In two independent experiments, five guinea pigs were immunized in the aerosol delivery system with 0.01 LD of *L. pneumophila* and five additional guinea pigs were exposed to buffer (controls). 28 d later, all animals were exposed simultaneously to an aerosolized dose of 10^8 CFU/ml of *L. pneumophila*. One immunized and one control guinea pig were then killed at 1, 6, 24, 48, and 72 h after exposure. The animals were soaked in Linbro 7X cleaning solution (Flow Laboratories, Inc.) and the right lung was removed from each animal using sterile technique. Each lung was placed in 10 ml of sterile EYB and ground up with a mortar

TABLE I

Fatality Rate of Guinea Pigs Administered Various Concentrations of L. pneumophila by Aerosol

Concentration of *L. pneumophila* in aerosol	Number of guinea pigs dead (per total exposed)
CFU/ml	
5×10^7	1/5
7.5×10^7	0/3
1×10^8	5/7
2.5×10^8	3/3
5×10^8	5/5

Guinea pigs were infected with aerosols containing the concentration of *L. pneumophila* indicated and the fatality rate at each concentration was determined.

and pestle. The suspension from each animal was cultured on CYE agar and CYE agar without cysteine (to rule out the presence of contaminating bacteria) as described (1).

Results

Aerosol Infection of Guinea Pigs with L. pneumophila. We exposed guinea pigs to various concentrations (10^4–10^9 CFU/ml) of live virulent *L. pneumophila* by aerosol and observed them for signs of infection. Animals exposed to $\geq 10^6$ CFU/ml consistently had fever with temperatures ≥40°C 2–5 d after infection. Animals exposed to $\geq 5 \times 10^7$ CFU/ml consistently displayed weight loss during this period. Animals given a lethal dose exhibited labored respirations, grunting, lethargy, ruffled fur, and shivering before death, which occurred 2–9 d after exposure, depending on dose. At necropsy, on gross examination, the lungs were hemorrhagic and congested or mottled with whitish yellow areas of consolidation. *L. pneumophila* was isolated in high concentrations from the lung and spleen, and also from the pericardium when pericardial effusion was present.

To determine the LD of *L. pneumophila*, defined here as the lowest concentration of *L. pneumophila* that was lethal to the majority of guinea pigs, we exposed guinea pigs to various concentrations of aerosolized *L. pneumophila* in several experiments and determined the rates of survival. Results were highly consistent from one experiment to the next. Animals exposed to aerosols containing $<10^8$ CFU/ml, infrequently died (Table I). In animals exposed to 10^8 CFU/ml, five of seven animals died; the two surviving animals exhibited typical premorbid signs of marked weight loss, labored respirations, and cyanotic extremities but they survived. Animals exposed to $\geq 2.5 \times 10^8$ CFU/ml all died (Table I). In view of these results, we defined the LD in this model as 10^8 CFU/ml. In subsequent experiments, all eight guinea pigs given 10^8 CFU/ml died; taken together with the previous data, this indicates that this dose is very near the LD_{100} for this system.

Guinea Pigs Exposed to Sublethal Concentrations of Aerosolized L. pneumophila Develop Humoral Immune Responses to the Bacterium. To determine whether guinea pigs exposed to sublethal concentrations of aerosolized *L. pneumophila* develop humoral immune responses, we measured the antibody titer to *L. pneumophila* of sera from these and control animals by the IFAb assay. Exposed

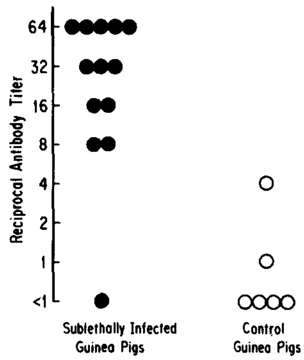

FIGURE 1. Guinea pigs sublethally infected with *L. pneumophila* develop a humoral immune response. Reciprocal IFAb titers in the serum of sublethally infected (*solid circles*) and control (*open circles*) guinea pigs were determined by the IFAb assay as described in the text.

animals but not controls developed significant antibody titers (Fig. 1). The median IFAb titer, expressed as the reciprocal of the highest positive dilution, from exposed guinea pigs was 32. Titers of control animals from <1 (negative with undiluted serum) to 4; the median titer was <1.

Guinea Pigs Exposed to Sublethal Concentrations of Aerosolized L. pneumophila Develop Cutaneous Delayed-type Hypersensitivity. To study cutaneous delayed-type hypersensitivity, we immunized guinea pigs with 10^4 to 10^7 CFU/ml of aerosolized *L. pneumophila* and 2 wk later, we assayed the response of these and control (nonimmunized) animals to intradermal injections of formalin-killed *L. pneumophila* in concentrations ranging from 10^5 to 10^{10} bacterial particles/ml (Table II, Exp. A).

Immunized animals had markedly greater areas of erythema and induration than control animals at both 24 and 48 h. Differences between immunized and control animals were particularly significant at 24 h with antigen concentrations of 10^7 and 10^8 formalin-killed *L. pneumophila*/ml: at 10^8 formalin-killed *L. pneumophila*/ml, immunized animals had 9.6 ± 2.4 mm of erythema and 7.3 ± 2.3 mm of induration, whereas controls had only 2.5 ± 2.5 mm of erythema and 0 mm of induration. At 10^7 formalin-killed *L. pneumophila*/ml, immunized animals had 7.8 ± 3.1 mm of erythema and 6.8 ± 3.2 mm of induration, whereas controls had 0 mm of erythema and induration.

In a second experiment, we immunized four guinea pigs with an aerosolized concentration of $\geq 10^6$ CFU of *L. pneumophila*/ml and skin-tested these and control animals 1 mo later with antigen concentrations of 10^7 and 10^8 formalin-killed *L. pneumophila*/ml (Table II, Exp. B). Again, immunized animals had markedly greater areas of erythema and induration at both 24 and 48 h to both antigen concentrations. Differences between immunized and control animals were particularly significant with an antigen dose of 10^7 formalin-killed *L. pneumophila*/ml. At 24 h, immunized animals exhibited 8.5 ± 2.7 mm of erythema and 5.8 ± 3.6 mm of induration, whereas control animals had 0 mm of erythema and 2 ± 2.8 mm of induration. At 48 h, immunized animals had 7.8 mm of erythema and 7.8 mm of induration, whereas controls had 0 mm of erythema and induration.

TABLE II

Skin Reactivity of Sublethally Infected and Control Guinea Pigs to Intradermal Formalin-killed L. pneumophila

Exp.	Time	Status of guinea pigs	n	Skin reaction	Extent of erythema and induration in response to indicated antigen concentrations (bacterial particles/ml)					
					10^{10}	10^9	10^8	10^7	10^6	10^5
	h				*mm*					
A										
	24	Immunized	8	Erythema	9.1 ± 5.6	9.0 ± 3.6	9.6 ± 2.4	7.8 ± 3.1	3.5 ± 3.9	3.5 ± 3.6
				Induration	9.5 ± 4.1	3.9 ± 3.4	7.3 ± 2.3	6.8 ± 3.2	0 ± 0	2.4 ± 3.2
		Control	2	Erythema	0 ± 0	4 ± 4	2.5 ± 2.5	0 ± 0	0 ± 0	0 ± 0
				Induration	6 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	48	Immunized	8	Erythema	7.1 ± 4.2	5.8 ± 4.6	5.6 ± 5.9	3.5 ± 3.8	0.9 ± 2.3	1.4 ± 2.4
				Induration	10.5 ± 2.2	7.3 ± 6.0	7.5 ± 3.8	4.6 ± 4.7	0.6 ± 1.7	0 ± 0
		Control	2	Erythema	5.5 ± 5.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
				Induration	6 ± 6	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Exp.	Status of guinea pigs	n	Skin reaction	Extent of erythema and induration in response to indicated antigen concentrations (bacterial particles/ml) at: 24 h		48 h	
				10^7	10^8	10^7	10^8
				mm			
B							
	Immunized	4	Erythema	8.5 ± 2.7	9.5 ± 1.7	7.8 ± 2.2	10.0 ± 1.6
			Induration	5.8 ± 3.6	10.0 ± 1.2	7.8 ± 2.2	10.3 ± 2.5
	Control	3	Erythema	0 ± 0	2.0 ± 2.3	0 ± 0	0 ± 0
			Induration	2.0 ± 2.8	3.3 ± 4.7	0 ± 0	6.3 ± 4.5

Guinea pigs were sublethally infected with *L. pneumophila*, and 2 (Exp. A) or 4 (Exp. B) wk later, they and control guinea pigs were skin-tested with formalin-killed *L. pneumophila*, as described in the text. Data given as means ±SD.

Splenic Lymphocytes from Guinea Pigs Recovered from a Sublethal Infection with L. pneumophila Proliferate in Response to L. pneumophila Antigens. To determine whether immunized guinea pigs expand a population of lymphocytes that recognize *L. pneumophila* antigens, we studied the proliferative response of splenic lymphocytes from these animals to formalin-killed *L. pneumophila*. In preliminary experiments, we studied the proliferative responses of splenic lymphocytes that were incubated with formalin-killed *L. pneumophila* for various incubation times (1–7 d) and at various concentrations of lymphocytes per milliliter. We obtained maximal responses when lymphocytes were at a concentration of 10^7 cells/ml and incubated with antigen for 2 d. We therefore used this concentration of lymphocytes and this incubation time in subsequent experiments.

Splenic lymphocytes from immunized guinea pigs consistently proliferated strongly in response to *L. pneumophila* antigens in comparison to splenic lymphocytes from control animals at antigen concentrations of 10^6 to 10^8 formalin-killed *L. pneumophila*/ml (Table III). Differences between immunized and control animals were most significant for antigen concentrations of 10^7 and 10^8 bacterial particles/ml. At 10^7 bacterial particles/ml, the mean determination for immunized animals was 7,727 ± 1,590 cpm (SE), whereas the mean determination for control animals was 2,397 ± 430 cpm; stimulation indices for immunized and control animals were 6.8 ± 1.2 and 1.7 ± 0.1, respectively. At 10^8 bacterial particles/ml, the mean determination for immunized animals was 15,957 ± 2,700

TABLE III

Proliferation of Splenic Lymphocytes from Sublethally Infected and Control Guinea Pigs in Response to Formalin-killed L. Pneumophila Antigens

Exp.	Immunized guinea pigs	[^{3}H]Thymidine incorporation by splenic lymphocytes incubated with *L. pneumophila* antigens at indicated concentration (bacterial particles/ml)						
		No antigen	10^6		10^7		10^8	
		cpm	cpm	SI*	cpm	SI	cpm	SI
A	A_1	1,619 ± 85	2,411 ± 130	1.5	5,880 ± 94	5.4	—	—
B	B_1	1,271 ± 75	1,975 ± 36	1.6	6,606 ± 270	5.2	14,933 ± 650	11.7
	B_2	485 ± 55	1,249 ± 29	2.6	2,879 ± 110	5.9	6,707 ± 200	13.8
	B_3	938 ± 50	4,291 ± 270	4.6	14,908 ± 1,020	15.9	20,265 ± 570	21.6
C	C_1	1,022 ± 67	3,953 ± 154	3.9	8,500 ± 260	8.3	22,418 ± 470	21.9
	C_2	457 ± 15	583 ± 15	1.3	1,092 ± 81	2.4	3,816 ± 63	8.3
D	D_1	1,532 ± 37	2,061 ± 89	1.4	3,908 ± 60	2.6	11,139 ± 220	7.3
	D_2	937 ± 45	2,412 ± 120	2.6	6,146 ± 280	6.6	19,204 ± 400	20.5
E	E_1	3,041 ± 530	12,096 ± 910	4.0	18,187 ± 780	6.0	31,887 ± 3,400	10.5
	E_2	994 ± 74	3,998 ± 830	4.0	9,160 ± 260	9.2	13,807 ± 1,100	13.9
Mean		1,230 ± 222	3,503 ± 976	2.8 ± 0.4	7,727 ± 1590	6.8 ± 1.2	15,957 ± 2,700	14.4 ± 1.6
	Control guinea pigs							
A	A_2	3,028 ± 170	2,555 ± 120	0.8	3,460 ± 170	1.1	—	—
B	B_4	630 ± 38	812 ± 12	1.3	1,125 ± 96	1.8	2,772 ± 160	4.4
C	C_3	901 ± 76	1,083 ± 60	1.2	1,453 ± 32	1.6	3,891 ± 270	4.3
D	D_3	1,334 ± 64	1,049 ± 250	0.8	2,558 ± 260	1.9	7,577 ± 2,050	5.7
E	E_3	1,772 ± 83	2,377 ± 83	1.3	3,388 ± 180	1.9	15,725 ± 580	8.9
Mean		1,532 ± 380	1,555 ± 320	1.1 ± 0.1	2,397 ± 430	1.7 ± 0.1	5,993 ± 2,700	5.8 ± 0.9

In each of five independent experiments (A–E), proliferation of splenic lymphocytes from one to three immunized animals and one control animal was studied. Splenic lymphocytes (10^7 cells/ml) were incubated in microtest wells at 37°C for 2 d without antigen or with formalin-killed *L. pneumophila* in concentrations of 10^6–10^8 bacterial particles/ml. The lymphocytes were then assayed for their capacity to incorporate [^{3}H]thymidine as described in the text; for each animal, the mean cpm ± SEM for three or four microtest wells is reported. At each antigen concentration, differences between SI of control and immunized guinea pigs are significant at $p = 0.02$ by a two-tailed t test.

* SI, stimulation index = (mean [^{3}H]thymidine incorporation (cpm) of lymphocytes incubated with antigen)/(mean [^{3}H]thymidine incorporation (cpm) of lymphocytes incubated without antigen).

cpm, whereas the mean determination for control animals was 5,993 ± 2,700; stimulation indices for immunized and control animals were 14.4 ± 1.8 and 5.8 ± 0.9, respectively.

Antigen concentrations of 10^9 bacterial particles/ml yielded strong proliferative responses in both immunized and control animals, and antigen concentrations of 10^{10} bacterial particles/ml yielded background levels of proliferation in all animals (data not shown).

Guinea Pigs Recovered from Sublethal Aerosol Infection with L. pneumophila Are Protected against Lethal Aerosol Challenge. To determine whether guinea pigs immunized by exposure to sublethal concentrations of *L. pneumophila* are protected against lethal challenge, we performed two large rechallenge experiments. In the first experiment, 33 guinea pigs were divided into three equal groups and exposed to EYB alone (control guinea pigs), 0.1 LD (10^7 CFU of *L. pneumophila*/ml), or 0.01 LD (10^6 CFU of *L. pneumophila*/ml). Fever of >40°C developed in all guinea pigs exposed to *L. pneumophila* by day 2 (21 guinea pigs) or day 3 (one guinea pig) and in none of the control animals. Weight loss occurred

TABLE IV

Survival Rates of Immunized and Control Guinea Pigs Administered a Lethal Aerosol Challenge of L. pneumophila

Exp.	Status of guinea pigs	Immunizing dose	n	Number of guinea pigs surviving (per number challenged at indicated dose [*LD*])			
				Buffer alone*	1	3	10
		LD					
1	Control	Buffer alone	11	1/1	0/3§	0/3‖	0/4
	Immunized	0.1	9‡	0/0	3/3§	3/3‖	0/3
		0.01	11	1/1	3/3	3/3	0/4
2	Control	Buffer alone	15	—	0/5¶	0/5**	0/5
	Immunized	0.01	15	—	5/5¶	5/5**	2/5

Guinea pigs initially were immunized with a sublethal dose of *L. pneumophila* (0.1 or 0.01 LD) or exposed to an aerosol containing buffer alone (controls). 28 d later, the animals were challenged with 1, 3, or 10 LD and the number of survivors was determined. Differences between immunized and control animals were evaluated by Fisher exact test, two-tailed. Differences between all immunized and control guinea pigs challenged with 1 or 3 LD significant in experiment 1 at $p < 0.0001$ and in experiment 2 at $p < 0.00005$. Differences between all immunized and all control guinea pigs challenged with 1, 3, or 10 LD, significant in Exp. 1 at $p < 0.005$ and in Exp. 2 at $p < 0.00001$.

* One control and one immunized animal were challenged with buffer alone in Exp. 1.

‡ 2 of 11 animals in this group died after receiving the 0.1-LD immunizing dose.

§ $p < 0.02$

‖ $p < 0.02$

¶ $p < 0.01$

** $p < 0.01$

in 6 of 11 guinea pigs in the 0.01 LD group, in 8 of 11 in the 0.1 LD group, and in 0 of 11 in the control group. Death occurred in two guinea pigs both in the 0.1 LD group, 3 and 12 d after exposure. 1 mo after the first exposure, we challenged the animals with either 1, 3, or 10 LD of *L. pneumophila* (Table IV). In animals given one LD, none of three control guinea pigs survived, but all six guinea pigs that had been sublethally infected with 0.1 or 0.01 LD survived. The three-LD challenge yielded the same results; i.e., none of three control guinea pigs and six of six sublethally infected guinea pigs survived. The 10-LD challenge was lethal for all guinea pigs in all three groups. As an additional experimental control, we exposed two guinea pigs (one in the control group and one in the group given 0.01 LD) to EYB alone in the rechallenge part of the experiment; as expected, both survived.

To confirm these results, we performed a second experiment in which we initially exposed 15 guinea pigs to 0.01 LD aerosolized *L. pneumophila* and 15 guinea pigs to aerosolized EYB alone (control guinea pigs). 15 of 15 guinea pigs exposed to *L. pneumophila* had temperatures >40°C by day 2 or 3, where 0 of 15 control guinea pigs had elevated temperatures. 11 of 15 guinea pigs exposed to *L. pneumophila* had some weight loss as did 6 of 15 controls. No guinea pig manifested ruffled fur or labored respirations. 1 mo after the first exposure, we exposed these animals to 1, 3, or 10 LD of aerosolized *L. pneumophila* as in the previous experiment (Table IV); 0 of 10 control animals survived challenge with one or three LD, whereas 10 of 10 immunized animals survived these doses.

With the 10-LD challenge, none of five control animals survived, whereas two of five immunized animals survived. Interestingly, in these experiments, guinea pigs in the immunized groups but not control groups had fever on the first day after challenge. In the first experiment, all 12 immunized guinea pigs had temperatures of >40°C (mean 40.7 ± 0.36) on the first day after one- or three-LD challenge, whereas none of six control guinea pigs had a temperature of >40°C (mean 39.2 ± 0.45) after the same challenge. Similarly, in the second experiment, 14 of 15 immunized guinea pigs had fever on the first day after challenge (mean 40.6 ± 0.46), whereas 0 of the 15 control animals was febrile (mean 39.12 ± 0.46). The fever in immunized animals was transient and subsided often by the second day after challenge. In the first experiment, for example, only 1 of 10 guinea pigs (temperature could not be taken in 2) was febrile on the second day after challenge.

Guinea pigs in both the immunized and control groups lost weight after challenge. In the first experiment, 10 of 12 immunized animals and 6 of 6 control animals exhibited weight loss after challenge with one of three LD. In the second experiment, all immunized and control animals displayed weight loss to challenge with these doses.

Guinea pigs in both the immunized and control groups exhibited other signs of illness in response to challenge with one or three LD, although the frequency was higher in the control group. The most frequently observed sign was labored respirations; this occurred in 7 of 12 immunized animals and 6 of 6 controls. Ruffled fur was observed in 1 of 12 immunized animals and 3 of 6 controls.

Immunized Guinea Pigs Limit L. pneumophila Multiplication and Clear L. pneumophila from Their Lungs. To determine the fate of *L. pneumophila* in immunized guinea pigs, we challenged five guinea pigs recovered from sublethal infection (0.01 LD) and five control guinea pigs with a one-LD aerosol, killed them 1–72 h later, and determined CFU of *L. pneumophila* in their right lung. We performed two independent experiments (Fig. 2). At 1 and 6 h after aerosol exposure, immunized and control guinea pigs had comparable numbers of CFU of *L. pneumophila* in their lungs in both experiments. By 24 h, the number of CFU markedly increased in both the immunized and control animals tested although the immunized animals had fewer bacteria (0.5 log fewer in the first experiment and 1.5 log fewer in the second experiment). Thereafter, CFU in control animals increased further to 4 logs above the 1-h level of infection, whereas CFU in immunized animals decreased in both experiments. At 72 h, CFU in the immunized animals had fallen to approximately the 1-h level of infection which was 4.5 log below the level in the control guinea pigs at this time point in both experiments; in the first experiment, the control guinea pig died before necropsy at 72 h.

Discussion

We have demonstrated that sublethal infection with *L. pneumophila* produced protective immunity in vivo against this intracellular pathogen. Moreover, we have demonstrated that sublethal infection with *L. pneumophila* by the airborne route, the natural route of infection, protects against lethal challenge by this

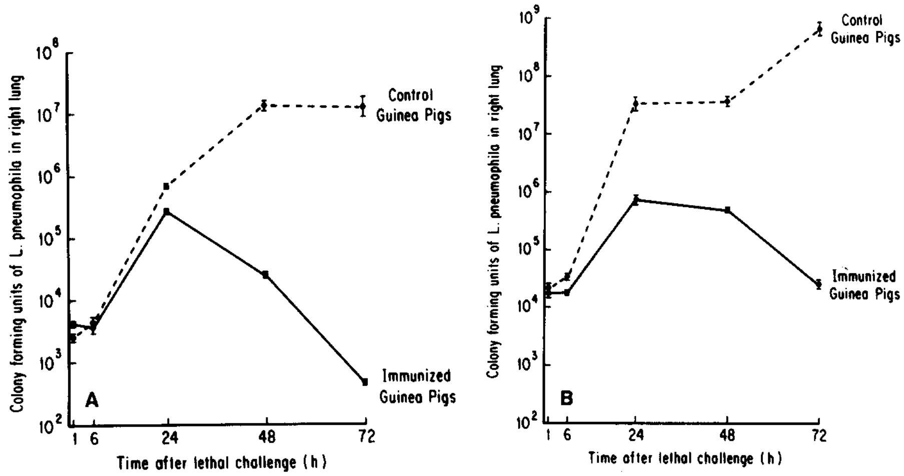

FIGURE 2. Immunized guinea pigs clear *L. pneumophila* from their lungs when challenged with a lethal dose. In two independent experiments (*A* and *B*), immunized and control guinea pigs were challenged with a lethal dose of *L. pneumophila* and sacrificed 1–72 h later. CFU of *L. pneumophila* in the right lung of each guinea pig was determined as described in the text. Each point represents the mean ± SEM of two independent measurements from the same lung sample.

route of infection. The development of protective immunity parallels the development of humoral and cell-mediated immune responses.

Previous studies have failed to demonstrate protective immunity to *L. pneumophila* in vivo (12, 13). We believe this is because the challenge doses in those studies were so high that they overwhelmed the capacity of the immune system to counteract the challenge. In our study, sublethally infected guinea pigs were protected against one or three times the lowest lethal dose that killed nearly all guinea pigs (~LD_{100}), but these animals were not protected against 10 times that dose. In the study by Baskerville et al. (12), sublethally infected guinea pigs were challenged with 10 times the LD_{50} and not protected. In the study by Eisenstein et al. (13), guinea pigs surviving a previous infection were challenged with 44 times the LD_{50} and were not protected.

In terms of lethality, the challenge doses we used, one and three times the ~LD_{100}, were still very high in comparison to the doses encountered by humans in epidemics of Legionnaires' disease. In most epidemics, the fatality rate has been ~15% in untreated cases. This dose would be roughly equivalent to 0.1 LD in our system, i.e., 1/30 the highest challenge dose used. Thus, the protective immunity induced by sublethal infection is very strong.

Immunized animals but not controls exhibited pyrexia on the first day after challenge. This likely reflects an early inflammatory response to *L. pneumophila* in the immunized animals. In the study by Baskerville et al. (12), immunized guinea pigs challenged with 10 times the LD_{50} died sooner than controls, even though the bacterial counts in the immunized animals were less than that of controls. It is possible that an early inflammatory response coming immediately after an overwhelming challenge resulted in the more rapid demise of these animals.

Cutaneous delayed-type hypersensitivity to *L. pneumophila* antigens developed in guinea pigs immunized by the aerosol route in our study. Cutaneous hypersensitivity to *L. pneumophila* antigens has previously been shown by Wong et al. (17) to develop in guinea pigs immunized by the intraperitoneal route with *L. pneumophila* and by the intradermal and intraperitoneal route with *L. pneumophila* antigens.

Lymphocyte proliferation to *L. pneumophila* antigens in vitro was also demonstrated in our study with splenic lymphocytes obtained from guinea pigs sublethally infected by the aerosol route. Splenic lymphocytes proliferated markedly 1–5 mo after guinea pigs were exposed to aerosols.

The right lungs of both immunized and control animals contained comparable numbers of bacteria immediately after exposure to a lethal concentration of *L. pneumophila*. In both the immunized and control animals, CFU did not significantly change between 1 and 6 h after aerosolization; this parallels in vitro data that *L. pneumophila* multiplication does not take place for the first few hours after infection of mononuclear phagocytes during which time a specialized ribosome-lined vacuole is formed (18). Between 6 and 24 h, CFU multiplied logarithmically in both control and immunized animals. However, between 24 and 48 h, bacterial CFU continued to increase logarithmically in the control animal but decreased in the immunized animal. This suggests that immune protective forces were marshalled and became effective during the first 24 h after exposure in the immunized animals. This may reflect the time required for the development of the activated state in mononuclear phagocytes exposed to lymphokines; such activated mononuclear phagocytes strongly inhibit *L. pneumophila* intracellular multiplication (4–6).

Since Legionnaires' disease is transmitted by the airborne route, aerosol infection of guinea pigs with *L. pneumophila* mimics the natural route of infection. Because the disease in guinea pigs closely resembles the disease in humans clinically and pathologically, aerosol infection of guinea pigs with *L. pneumophila* constitutes a superb animal model of Legionnaires' disease. As a number of other diseases of the lung are caused by intracellular pathogens that are transmitted by the airborne route (e.g., tuberculosis, psittacosis, Q fever, histoplasmosis, and tularemia), this model may also serve as an excellent one for studying general principles of host defense against lung infections caused by intracellular pathogens.

Summary

We have employed the guinea pig model of *L. pneumophila* infection, which mimics Legionnaires' disease in humans both clinically and pathologically, to study humoral and cell-mediated immune responses to *L. pneumophila* and to examine protective immunity after aerosol exposure, the natural route of infection. Guinea pigs exposed to sublethal concentrations of *L. pneumophila* by aerosol developed strong humoral immune responses. By the indirect fluorescent antibody assay, exposed guinea pigs had a median serum antibody titer (expressed as the reciprocal of the highest positive dilution) of 32, whereas control guinea pigs had a median titer of <1. Sublethally infected (immunized) guinea pigs also developed strong cell-mediated immune responses. In response to *L. pneumophila*

antigens, splenic lymphocytes from immunized but not control animals proliferated strongly in vitro, as measured by their capacity to incorporate [^{3}H]thymidine. Moreover, immunized but not control guinea pigs developed strong cutaneous delayed-type hypersensitivity to intradermally injected *L. pneumophila* antigens.

Sublethally infected (immunized) guinea pigs exhibited strong protective immunity to *L. pneumophila*. In two independent experiments, all 22 immunized guinea pigs survived aerosol challenge with one or three times the lethal dose of *L. pneumophila* whereas none of 16 sham-immunized control guinea pigs survived ($p < 0.0001$ in each experiment). Immunized guinea pigs were not protected significantly from challenge with 10 times the lethal dose. Immunized but not control animals cleared the bacteria from their lungs.

This study demonstrates that guinea pigs sublethally infected with *L. pneumophila* by the aerosol route (*a*) develop strong humoral immune responses to this pathogen, (*b*) develop strong cell-mediated immune responses and cutaneous delayed-type hypersensitivity to *L. pneumophila* antigens, (*c*) are protected against subsequent lethal aerosol challenge, and (*d*) are able to clear the bacteria from their lungs. The guinea pig model of *L. pneumophila* pulmonary infection is as an excellent one for studying general principles of host defense against pulmonary infections caused by intracellular pathogens.

We are grateful to Dr. Paul Edelstein for his advice and assistance in the use of the guinea pig model and to Ms. Barbara Jane Dillon and Ms. Debora S. Gloria for their generous technical help.

Received for publication 19 November 1986.

References

1. Horwitz, M. A., and S. C. Silverstein. 1980. Legionnaires' disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. *J. Clin. Invest.* 65:441.
2. Horwitz, M. A., and S. C. Silverstein. 1981. Interaction of the Legionnaires' disease bacterium (*Legionella pneumophila*) with human phagocytes. I. *L. pneumophila* resists killing by polymorphonuclear leukocytes, antibody, and complement. *J. Exp. Med.* 153:386.
3. Horwitz, M. A., and S. C. Silverstein. 1981. Interaction of the Legionnaires' disease bacterium (*Legionella pneumophila*) with human phagocytes. II. Antibody promotes binding of *L. pneumophila* to monocytes but does not inhibit intracellular multiplication. *J. Exp. Med.* 153:398.
4. Horwitz, M. A. 1983. Cell-mediated immunity in Legionnaires disease. *J. Clin. Invest.* 71:1686.
5. Nash, T. W., D. M. Libby, and M. A. Horwitz. 1984. Interaction between the Legionnaires' disease bacterium (*Legionella pneumophila*) and human alveolar macrophages: influence of antibody lymphokines, and hydrocortisone. *J. Clin. Invest.* 74:771.
6. Horwitz, M. A., and S. C. Silverstein. 1981. Activated human monocytes inhibit the intracellular multiplication of Legionnaires' disease bacteria. *J. Exp. Med.* 154:1618.
7. Davis, G. S., W. C. Winn, Jr., D. W. Gump, J. E. Crayhead, and H. N. Beaty. 1982.

Legionnaires' pneumonia after aerosol exposure in guinea pigs and rats. *Am. Rev. Respir. Dis.* 126:1050.

8. Davis, G. S., W. C. Winn, Jr., D. W. Gump, and H. N. Beaty. 1983. The kinetics of early inflammatory events during experimental pneumonia due to *Legionella pneumophila* in guinea pigs. *J. Infect. Dis.* 148:823.
9. Baskerville, A., R. B. Fitzgeorge, M. Broster, P. Hambleton, and P. J. Dennis. 1981. Experimental transmission of Legionnaires' disease by exposure to aerosols of *Legionella pneumophila. Lancet.* ii:1389.
10. Katz, S. M., and S. Hashemi. 1982. Electron microscopic examination of the inflammatory response to *Legionella pneumophila* in guinea pigs. *Lab. Invest.* 46:24.
11. Berendt, R. F., H. W. Young, R. G. Allen, and G. L. Knutsen. 1980. Dose response of guinea pigs experimentally infected with aerosols of *Legionella pneumophila. J. Infect. Dis.* 141:186.
12. Baskerville, A., R. B. Fitzgeorge, J. W. Conlan, L. A. E. Ashworth, D. H. Gibson, and C. P. Morgan. 1983. Studies on protective immunity to aerosol challenge with Legionella pneumophila. *Zentrabl. Bakteriol. Parasitenkd. Infectionskr. Hyg. Abt. 1 Orig. A.* 255:150.
13. Eisenstein, T. K., R. Tamada, J. Meissler, A. Flesher, and H. C. Oels. 1984. Vaccination against *Legionella pneumophila*: serum antibody correlates with protection induced by heat-killed or acetone-killed cells against intraperitoneal but not aerosol infection in guinea pigs. *Infect. Immun.* 45:685.
14. Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* 99:167.
15. Wilkinson, H. W., B. J. Fikes, and D. D. Cruce. 1979. Indirect immunofluorescence test for serodiagnosis of Legionnaires disease: Evidence for serogroup diversity of Legionnaires' disease bacterial antigens and for multiple specificity of human antibodies. *J. Clin. Microbiol.* 9:379.
16. Horwitz, M. A., and S. C. Silverstein. 1980. Influence of the *Escherichia coli* capsule on complement fixation and on phagocytosis and killing by human phagocytes. *J. Clin. Invest.* 65:82.
17. Wong, K. H., P. R. B. McMaster, J. C. Feeley, R. J. Arko, W. O. Scalla, and F. W. Chandler. 1980. Detection of hypersensitivity to *Legionella pneumophila* in guinea pigs by skin test. *Curr. Microbiol.* 4:105.
18. Horwitz, M. A. 1983. Formation of a novel phagosome by the Legionnaires' disease bacterium (*Legionella pneumophila*) in human monocytes. *J. Exp. Med.* 158:1319.